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COMPUTER SUPPORT OF HEMOGLOBIN AND BLOOD RESEARCH

FINAL REPORT

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EDWARD C. DELAND

SEPTEMBER 1, 1992

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Los Angeles, California 90024-1406

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It is well documented that the Adair constants, a_j, in the saturation equation change, sometimes radically, with changing experimental conditions. Winslow[1], et al., show the dependence of the Adair constants on pH, PCO_2, and DPG; other affectors are Cl^- and temperature. We propose a method to incorporate these affectors into the Adair equation in order to predict the saturation under variable conditions. Previous theoretical models of hemoglobin function generally have been limited in scope because the conceptual complexity of the protein does not readily admit explicit modeling. Here we describe a method that allows systems of arbitrary complexity to be modeled readily, and a method to derive an exact relationship between affector concentration and the Adair parameters.

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19. Abstract (continued)

This work, supported by the Blood Research Group at Letterman Army Institute of Research, has been focused upon the study of isoionic and cross-linked hemoglobin. With the benefit of a complex model it is possible to show, for example, that the Adair parameters are not constant even over the range of a blood saturation curve. Nevertheless, the Winslow[2] and Vandegriff[3] parameters, are probably accurate for zero-DPG experiments in which other affectors are held constant. While such facts may be intuitively sensible, there is also a physical-chemical explanation.

We also wish to demonstrate the increasingly necessary relationship between sharply focused laboratory data and critical analysis. The explicit, computerized detail depicting molecular hypotheses are now sufficiently complex that detailed, focused questions can be formulated for the laboratory, and conversely, the laboratory can formulate more complicated molecular hypotheses. For example, explicit simulations of the DPG pocket use data from Hb derivatives, such as Manning[4], and detailed binding, such as Tyuma[5]. These data can now be incorporated in a simulation for analysis of the implied molecular hypotheses.

(Key words: Blood, hemoglobin, mathematical simulation, blood substitutes)

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[1]Winslow, R.M., M. Samaja, N.J. Winslow, L. Rossi-Bernardi, R.I. Schrager, *Simulation of Continuous Blood O_2 Equilibrium Curve over Physiological pH, DPG, and PCO_2 Range*, J. Appl. Physiol: Respirat. Environ. Exercise Physiol., Vol. 54, No. 2, 1983, pp. 524-529.

[2]Winslow 1983, ibid.

[3]Vandegriff, K.D., F. Medina, M.A. Marini, and R.M. Winslow, *Equilibrium Oxygen Binding to Human Hb Cross-linked Between Alpha-chains by bis(3,5-dibromosalicyl) Fumarate*, J. Biol. Chem., Vol. 264, 1989, pp. 17824-17833.

[4]Nigen, A.M., J.M. Manning, and J.O. Alben, *Oxygen-linked Binding Sites for Inorganic Anions to Hemoglobin*, J. Biol. Chem., Vol. 255, No. 12, 1980, pp. 5525-5529.

[5]Imaizumi, K., K. Imai, and I. Tyuma, *The Linkage between the Four-step Binding of Oxygen and the Binding of Heterotropic Anionic Ligands in Hemoglobin*, J. Biochem., Vol. 86, 1979, pp. 1829-1840.

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THE ADAIR EQUATION REVISITED: MODELS OF HEMOGLOBIN STRUCTURE AND FUNCTION

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September 1, 1992

This work was supported by the Blood Research Group, Letterman Army Institute of Research, Presidio of San Francisco, Col. R. M. Winslow, Director.

THE ADAIR EQUATION REVISITED: MODELS OF HEMOGLOBIN STRUCTURE AND FUNCTION

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1. Introduction

Mathematical models of hemoglobin structure and function have been devised for many years to aid the study of research hypotheses. These models generally have been limited in scope because the conceptual complexity of the protein does not readily admit explicit modeling. Here we describe a method that allows systems of arbitrary complexity to be modeled readily and we apply it to problems from the work in the LAIR Blood Research Laboratory[1] on isoionic and cross-linked hemoglobin.

A central problem in hemoglobin research over the years has been the accurate determination of the oxygen binding parameters at the four sites in the complex protein, This apparently simple task of fitting a function to the observed saturation data curve has, however, become increasingly complicated. Because of the intrinsic property of the protein-oxygen system known as cooperativity (or heme-heme interaction, as first designated by Bohr in 1903[2]) the very definition of the observed binding parameters comes into question and has led researchers to the necessity of understanding the myriad ancillary structural and functional properties of the protein. A theoretical simulation can aid this research activity by displaying reseearch hypotheses.

It is possible to calculate the equilibrium distribution of reaction products of a complex system by finding the minimum of the Gibb's free energy function for the set of reactions at the given temperature and pressure. Further, if some of the reactions parameters or constants are unknown, they may be approximated by fitting the calculated result to observed data. Such a method for blood research was proposed by Dantzig[3] in 1966 based upon previous work be White, et al.[4] and this work evolved into the current computer program. At about the same time, several other laboratories developed programs using the same optimization principle for the study of inorganic chemical systems.[5,6,7] Prior application in this laboratory have primarily been in studies of whole blood and in whole body fluid and electrolyte balance.[8,9,10,11]

These model constructs of protein and blood detail are called "isomorphic" models because they profess to be one-to-

one copies of the hypothetical system under study, reaction for reaction, though, of course, the real system may be dauntingly more complicated. They are limited however by our knowledge of the system and availability of critical data rather than computational difficulty.

With the benefit of an explicit complex model, multivariate relationships can arise to be examined that are ordinarily fairly difficult to perceive. One of these is the known variability of the Adair "constants" under a variety of different experimental conditions. It is now possible to show, for example, that he Adair parameters are not "constant" over the range of a blood saturation curve, but that the "constant" Vandegriff parameters[12], nevertheless, are probably accurate for isoionic, constant-pH, cross-linked $\alpha\alpha$Hb. While such facts may be intuitively sensible, there is also a physical-chemical explanation that we will develop.

Here, we begin a mathematical analysis and review of the problem with the goal of clarifying certain basic concepts relating to the oxygenation parameters. Subsequent notes will deal with other aspects of the problem, e.g., allostery. We also wish to demonstrate the increasingly necessary relationship between sharply focused laboratory data and critical analysis. The explicit, computerized detail depicting molecular hypotheses are now sufficiently complex that detailed, focused questions can be formulated for the laboratory, and conversely, the laboratory can formulate more complicated molecular hypotheses.

2. Statement of the Problem

The simple S-shape of the hemoglobin saturation curve can, of course, be fit arbitrarily well by any number of functions, a sum of exponentials, for example. However, such a function, not being derived from fundamental biochemistry, will not help to explain why the protein produces such a curve. Researchers in hemoglobin have therefore tried to argue from fundamental principles. If we suppose, for example, that n oxygen molecules can attach to the hemoglobin each with the identical binding constant K as in the equation:

$$Hb + n\,O_2 \Leftrightarrow Hb(O_2)_n$$

then the oxygen saturation of Hb, y, can be written as the ratio of concentrations of liganded sites to the total number of sites:

$$y = \frac{Hb(O_2)_n}{Hb + Hb(O_2)_n} ,$$

or

$$y = \frac{Kp^n}{1 + Kp^n} , \qquad (1$$

where the chemical symbols indicate concentration, a convention we adopt for most of this paper. This equation, first proposed by Hill[13], in 1910, introduced two important concepts (in addition to the very idea of an abstract mathematical model based on chemical principles): the first, suggested by Hill, that the equilibrium constant, K, may be regarded as proportional to the probability of finding the protein occupied by oxygen, and the other that by adjusting just two fixed parameters it may be possible to "model" the observed saturation curve.

Hill's equation, particularly in its logarithmic form

$$Y = \log \frac{y}{1-y} = n \log p + \log K , \qquad (2$$

showing the relationship between n and the probability factor K, is still very much in use today, though we now know that just two parameters are not sufficient to describe the complexity of the saturation curve. In fact, we will show that perhaps several dozen parameters may be necessary, and can be derived.

At a given moment, for reasons to be discussed, most of the tetrameric hemoglobin molecules in solution with high probability will be either completely oxygenated (oxy) or completely deoxygenated (deoxy). But, the tetramer, being a complex system of variable bonds, linkages, and critical bindings, can assume a vast number of intermediate states depending upon ambient variations of, say, pH, PCO_2, or Cl^- concentration. By this view, the probability of finding the molecule in a particular state is an aggregate function of the microscopic probabilities for each binding, linkage, or bonding that can occur locally. We speak of the global properties of the solution, such as O_2 saturation or bound DPG concentration, because the law of large numbers allows such an overall summation or averaging operation. In particular, the probability of finding a given molecule in a certain oxygenated state can be altered by changing any of the ancillary microscopic probabilities, such as the

buffering of protons, as might occur during a saturation curve experiment.

We will show that it is possible to derive a mathematical model that incorporates the necessary, effective microscopic probabilities of internally consistent theoretical hypotheses, but this does imply that quite a few parameters must be postulated and validated in conjunction with the laboratory.

3. The Proliferation of Parameters: The Four Parameter Model

After the historical determination that Hb has exactly four oxygen binding sites, Adair[14] developed his famous 4-parameter formula for calculating the saturation curve:

$$y = \frac{a_1 p + 2a_2 p^2 + 3a_3 p^3 + 4a_4 p^4}{4(1 + a_1 p + a_2 p^2 + a_3 p^3 + a_4 p^4)} \qquad (3$$

where $a_i = \prod_1^i k_j$, $i=1,\ldots,4$, the k_j are the sequential oxygen equilibrium constants at the four sites, and p is the pressure of oxygen in mmHg. The Adair equation, the basis of considerable subsequent work, produces a saturation curve having certain necessary qualitative aspects as well as a rational, that is chemical, theoretical base. However, it apparently has two limitations: first, it still gives puzzling characteristic errors with respect to an observed saturation curve, and, second, it does not in itself explain the observed shifts of the curve with changes of pH, PCO_2, or temperature.

From the theoretical equation, Eqn. 3, we can derive the corresponding equation used by the laboratory to measure the saturation. Since

$$Hb + jO_2 \Leftrightarrow Hb(O_2)_j \ , k_j, \ j=1,\ldots,4$$

or

$$Hb(O_2)_j = \frac{a_j p^j}{Hb}, \qquad (4$$

where we have changed the units of oxygen concentration to mmHg, we can substitute in Eqn. (3 to get:

$$y(p) = \frac{HbO_2 + 2Hb(O_2)_2 + 3Hb(O_2)_3 + 4Hb(O_2)_4}{4(1 + HbO_2 + Hb(O_2)_2 + Hb(O_2)_3 + Hb(O_2)_4)}, \qquad (5$$

which is the ratio of oxygenated sites to all sites on the Hb at a given pressure, p.

The laboratory, by Van Slyke, optical or some other means, measures and reports as observed data the numerator of Eqn. 5. In these notes we are asking why the calculated Eqn. 3 does not yield the same result as the observed Eqn. 5? A simple answer is that Eqn. 3 involves the aj explicitly, and Eqn. 5 does not, except implicitly in the protein itself. The subtlety of the protein is not represented in Eqn. 3., and its error with respect to the observed curve must therefore in some way be reflected in the variability or the structure of the aj. Specifically, then, if the aj are not constant under a variety of laboratory conditions, exactly (mathematically) why and how do the aj change with pH or other ambient, protein variables?

A theoretical model of Hb function should minimally reproduce observed data; a better model would also predict *and explain* laboratory results over the range of laboratory conditions. Such a model is known as a "general" model (rather than specific to a certain set of conditions).

4 The Intrinsic vs. Observed Constants

With Eqn. 3, it is necessary to refit the equation to the observed data after, say, a change in ambient pH, which results in a new set of binding parameters, aj.[15,16] The question arises, is it possible for the parameters automatically to be adjusted intrinsically in the model as in the protein? Again, it is useful to consider the "Adair constants", aj, as proportional to the probability of finding a particular site occupied, and then to question what reasonably can affect these probabilities. The commonly considered affectors are pH, CO_2, temperature, phosphate binding, Cl^-, and the allosteric and stereochemical properties of the protein. The principles upon which these affectors might work are reasonably well known chemical concepts such as competition for the same binding site or altering a local charge field.[17,18,19,20,21]; while the details for a particular protein such as Hb are still the objectives of theoretical hypotheses. The task is to incorporate these affectors into the mathematics using just basic chemical principles in such a way that the "observed" or effective aj are modified just as in the real protein under varying experimental conditions. We

do this by simulating theoretical hypotheses and validating against laboratory data.

Initial hypotheses in this direction invoked cooperativity of the protein[22], which essentially just suggests that as the sequential oxygens are bound, each binding affects the local environment to alter the conditions under which the next will be bound. Thus, although the a_j may be nearly equal on the reduced molecule, they are effectively quite different as oxygenation proceeds. (This concept of the tetramer also raises the question of whether the individual a_j of the monomers are modified in the formation of dimers and the tetramer. This, question, in turn, raises the probability that one of the oxygen sites in the tetramer has greatest affinity and will be the first to bind O_2[23,24,25], an important problem for building a model.) As to exactly how the a_j are modified: "It must be assumed that these interactions are mediated by some kind of molecular transition (allosteric transition) which is induced or stabilized in the protein when it binds an 'allosteric ligand'", Monod, et al.[26].

The emergent concept is that the individual monomers have an intrinsic binding constant for oxygen and, whether α or β, they may be nearly equal or differ by a small factor (Baldwin, 1979), but bound into the tetramer the observed binding constants may be quite different and quite variable depending upon experimental circumstances. In particular, the different values of the observed (or effective parameters to fit an observed curve) are a consequence of the affectors above operating on (modifying) the intrinsic constant. The idea is that the intrinsic constants remain the same, but we observe quite different constants, and that the operator that produces the effective or observed constant can be calculated.

We note in passing that by implication if we can construct (simulate) the functional operator and we know the observed constant, an inverse process will calculate the intrinsic constant.

The mathematical goal, here, is to explicitly detail these phenomena by incorporating additional molecular hypotheses into the fundamental Adair equation. Pauling was the first to show the explicit relationship of an affector (pH) in a mathematical model. Thus, Pauling[27], in 1935, developed a modified Adair equation with the following form:

$$y = \frac{Kp + (2\alpha+1)K^2p^2 + 3\alpha^2K^3p^3 + \alpha^4K^4p^4}{1 + 4Kp + (4\alpha+2)K^2p^2 + 4\alpha^2K^3p^3 + \alpha^4K^4p^4}, \qquad (6$$

where α is a stabilizing ($\alpha>1$) interaction coefficient between adjacent hemes, and the single oxygenation constant is a parameter. Pauling, further recognizing that the Adair "constants" can not be constant over the entire curve introduced a function of pH to relate the intrinsic and observed binding constant:

$$K' = K \frac{(1+\beta A/H^+)^2}{(1+A/H^+)^2} \qquad (7$$

where β is an interaction constant and A the proton ionization constant.

While this model does not yet explain or detail just how the α and β coefficients work to modify the binding constants at the molecular level, it has an explicit awareness that the Adair "constants" are not constant over the range of PO_2 of a saturation curve (or under varying experimental conditions) because, for example, the pH changes.

Several subsequent models of the saturation curve have been offered; two, in particular, specifically incorporate the stereochemical *affector and will be important* in the present discussion, the mwc model:

$$y = \frac{(LK_t p(1+K_t p)^3 + K_r p(1+K_r p)^3}{(L(1+K_t p)^4 + (1+K_r p)^4} \qquad (8$$

of Monod, Wyman, and Changeaux (Monod 1965) and the cooperon model of DiCera, Robert, and Gill[28]:

$$y = \frac{(L(K_t p+\gamma K_t^2 p^2)+2K_r p(1+K_r p)^3}{(L(1+2K_t p+\gamma K_t^2 p^2)+2(1+K_r p)^4} \qquad (9$$

The parameter L designates a change in free-energy of the tetrameric structure as the hemoglobin undergoes a step allosteric transition during the sequence of oxygenation. K_t and K_r are the oxygen binding constants at all four hemes in the two states.

The two probable states of the molecule, designated R and T, are reversibly accessible and they differ by the distribution of energy throughout the inter-dimeric bonds and, indeed, throughout the entire tetramer. As a result, the probability for oxygen binding at the stereolabile sites (the "allosteric effect) is altered by the factor L as the transition occurs. This mathematical idea is similar to Pauling's in that an empirical constant is introduced to account for and correct persistent, characteristic errors of previous theoretical models. The new parameters are also based upon or derived from a novel molecular hypotheses. A drawback is that the parameter is empirical rather than arising from first principles, say, for example, the calculation of charge distribution. While this is sometimes necessary (because of molecular complexity) it runs the same risk of lack of generality as the Adair theory, and , indeed, this has proved to be the case (discussed in Section 8).

Subsequently, Ackers, in a series of papers, devised chemical and thermodynamic formulations of these complex phenomena and proposes distinct theoretical hypotheses to explain observational data. In particular, for example in Ackers[29] and Smith[30], the authors propose specific functional relationships among the affectors and the observed binding coefficients for oxygen, and they offer considerable experimental verification. Finally, in 1987, Ackers[31], et al., using data from that laboratory, show that the earlier models, Eqns. 8 and 9, are not consistent with observations and propose a three-state molecular hypothesis.

Trial models of these hypotheses have been devised, and we have proposed critical tests to aid distinguishing among them.

5. The Adair Isomorphic Model

One consequence of the previous work has been considerable attention focused on the intermediate states of hemoglobin during oxygenation. Such information would aid theoretical design, but in the laboratory direct observation of these compounds and the transition states of the tetramer has proved difficult (except for carbonmonoxy-hemoglobin) so that validation of a particular molecular hypotheses generally awaits critical data. Of course, a mathematical simulation of the protein function will necessarily calculate the quantity and variety of intermediate compounds resulting from a particular molecular hypotheses, however complicated.

By a "mathematical model" we mean a list of biochemical reactants and products hypothesized to be relevant and significant for determining the observed function of the protein, plus relationships or conditions among the products, such as stereochemical constraints, that may preclude or alter the probability of an event. Such a model could be subjected to the exact same protocol, say, titration, as in the laboratory and the calculated results displayed for comparison and analysis. Should the results be identical to the observed, this does not necessarily validate the underlying molecular hypotheses, since another model may do as well. But if the results are not in agreement with the laboratory, either the hypotheses require more thought, or the particular model must be redesigned to truly reflect the theoretical detail.

In this note series, we will show models of several distinct molecular hypotheses, beginning with the early Adair (1925) and Roughton[32] models. Both the Adair and Roughton models were devised before the remarkable work of Chanutin and Churnish[33] (and simultaneously Benesch and Benesch[34]) demonstrated the importance of 2,3-DPG, so these models do not include an effect of the phosphate binding.

The Adair molecular hypothesis of 1910 was simply that four oxygens bind to one HB. and we therefore have the simple isomorphic structure of Table 1.

The Adair model is a two phase system in which, as Table 1a shows, 4.9 mM of Hb are dissolved in one liter of water, about the concentration in red cells (1 l H_2O = 55.137 moles, 37° C). The gas solubility constants are shown in the gas phase, Table b. The "multipliers" indicate that this experiment will use 1 liter of solution and 10^5 moles of a gas mixture having 26.6 mmHg O_2 (pp = 0.035) and saturated with water (46.4 mmHg at 37° C). And since the protein added is a "chemically pure", neutral molecule, some H^+ is removed to make the protein in solution isoionic, pH = 7.32 at 50% saturation. HCO_3^- is included for a later experiment, but here since PCO_2 is very small in the gas, bicarbonate will be negligible.

A typical calculation result is given in Table 1c, where the Hb has 50% saturation. In Table 1c, the quantities of the output species are listed in moles; they could be calculated in any concentration unit. Since the amount of solvent water input was 1 liter, the output is one liter plus the volume of the protein (about 50 ml/mM Hb) so that the

a. ADAIR MODEL FOR Hb OXYGENATION		
INPUT REACTANTS:	**Input (moles)**	**Gas (pp)**
Multipliers:	1.0	1.0 E+5
O2	3.923 D-06	3.500 D-02
CO2	1.948 D-10	1.315 D-08
N2	8.066 D-04	9.377 D-01
H2O	5.513 D+01	6.107 D-02
H+	-2.286 D-02	
Hb	4.901 D-03	

b. REACTION EQUATIONS FOR ADAIR MODEL				
REACTION PRODUCTS:	**REACTION CONSTANT**	**REACTANTS**		
Gas Phase				
O2	0.026	1.0 O2		
CO2	0.0440	1.0 CO2		
N2	0.0146	1.0 N2		
H2O	2.79569	1.0 H2O		
Hb Solution				
O2	0.0	1.0 O2		
CO2	0.0	1.0 CO2		
N2	0.0	1.0 N2		
H2O	0.0	1.0 H2O		
H+	0.0	1.0 H+		
OH-	13.0958	1.0 H2O	-1.0 H+	
HCO3-	6.13	1.0 CO2	1.0 H2O	-1.0 H+
Hb	0.	1.0 Hb		
HbO2	-1.222	1.0 Hb	1.0 O2	
$Hb(O2)_2$	-2.428	1.0 Hb	2.0 O2	
$Hb(O2)_3$	-3.617	1.0 Hb	3.0 O2	
$Hb(O2)_4$	-5.184	1.0 Hb	4.0 O2	

Table 1a,b. A Computer-based model of the Adair molecular hypothesis: four Oxygen molecules per tetramer (37° C, PCO2, DPG, CL^- = 0). Table a lists the reactants to simulate 1 liter of 4.9 millimolal Hb solution. Table b lists the expected chemical reactions with their pKs. The reaction products are listed in the left column followed by the reaction equilibrium constants (pKs or solubilities), and followed by the reactants in the columns to the right (see text for details).

mole values are actually in molal units. Elsewhere in this note, we deduct from the input an amount of water equal to the protein volume, so that the output species are in molar units. The amount of water required to make HCO_3^- is added, but the water for initial solvation of the protein, perhaps 1000 molecules per tetramer, is presumed included with the input protein.

CALCULATED PRODUCTS FOR ADAIR Hb MODEL			
Output Species:	**Units**	**Gas Phase** (pp)	**Hb Solution** (moles)
pH:			7.321
O2	MOLES	3.500 E+02	3.923 D-06
CO2	MOLES	1.315 E-03	1.948 D-10
N2	MOLES	9.376 E+04	8.066 D-04
H2O	MOLES	6.107 E+02	5.514 E+01
H+	MOLES		4.186 E-08
OH-	MOLES		3.090 E-07
HCO3-	MOLES		1.303 E-09
Hb	MOLES		1.589 E-03
HbO2	MOLES		8.500 E-04
$Hb(O2)_2$	MOLES		4.470 E-04
$Hb(O2)_3$	MOLES		3.352 E-07
$Hb(O2)_4$	MOLES		2.014 E-03

Table 1c. Calculated Output for the Adair Model of Table 1a,b (see text for details).

A simulation of Hb might consist of varying the PO_2 (and inversely the N_2 to maintain one atmosphere pressure) to calculate a saturation curve, as in the laboratory. The a_j can be determined by using this chemical model as an arbitrary function in standard regression programs. Fitting the model will give the same results as fitting the Adair equation itself because this is a complete, that is, a one-to-one, model of the Adair hypothesis. Figs. 1a,b are calculated from Eqn. 3, but could have been generated by using this model with the same a_j.

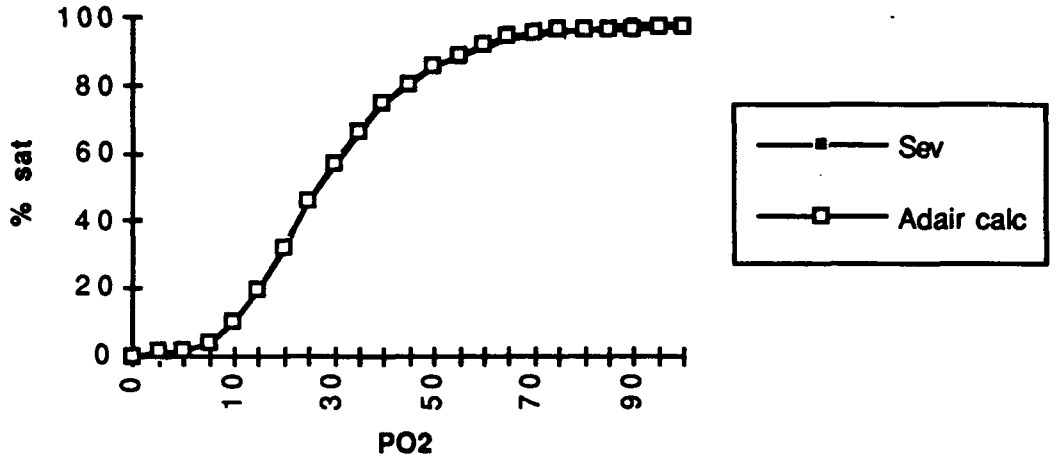

Fig 1a. Typical Adair equation fit to Severinghaus data.

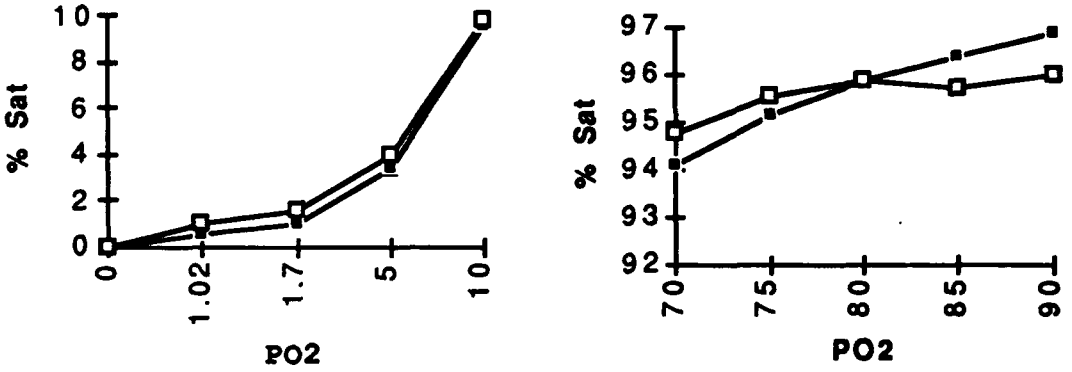

Fig. 1b. Expanded view of typical curve fit by the Adair Equation model of Table 1, where the open squares are calculated (see text for details).

The entire curve (Fig. 1a) does not clearly show the characteristic errors of the Adair equation. Typically, the calculated curve compared to the observed curve in blood, i.e., in the blood's full complement of affectors, shows too great an affinity for oxygen below about 10 mmHg PO_2, and, after crossing the observed curve at about 45 and 95 mmHg, too low an affinity above 95 mmHg. In Fig. 1b, two expanded portions of a calculated curve clearly show these "characteristic" errors using the "standard" (for 1966) blood saturation data of Severinghaus[35] and calculations from Eqn. 3.

A modern explanation for this rests upon the DPG effect addressed by the mwc and cooperon models. For example, calculations from the Adair model do not show the delayed first stage affinity (when DPG is bound to Hb) or the increased fourth stage affinity (when the probability of DPG binding is small). With respect to the model, this means that at the lower end of the curve, bound DPG decreases the effective value of a_1, and at the upper end of the curve, increases the effective values of a_3 and a_4. That linkage is not reflected explicitly in the Adair equation, so the a_j remain constant. If such functions could be built into the Adair model, the calculated curve would be delayed at the bottom and pushed to the left at the top, a counter-clockwise rotation, as with the mwc model, which would better fit the observed data.

If the goal is just to get a better Adair fit, laboratory conditions can be held constant. In experiments with isoionic HbaO, cross-linked protein, or even blood in which all affectors are held constant (and with DPG = 0), Vandegriff (1989), Winslow[36], and Winslow[37] get reasonably good fits with the Adair. Of course, the a_j do change when the experimental conditions are changed (as in Winslow 1983), a result that will be avoided with a more complex model.

Because of this and similar shortcomings, the Adair is expected to fail as a general model; four parameters are not enough to incorporate the complicated Hb functions. The mwc and cooperon models are attempts to cure this problem, and Vandegriff (1989) finds that in some circumstances they do give a better fit (discussed in Section 8). Generally, however, the same arguments apply to these models as apply to Adair, that the parameters, in this case L and γ are merely empirical rather than derived from biochemical principles.

6. The Roughton Isomorphic Model

The Roughton (1972) model explicitly includes the Bohr and Haldane effects linked to oxygenation so, if his molecular hypotheses are correct, the a_j will not change with changing experimental pH or PCO2. Specifically, Roughton proposed that CO_2 was bound at the N-terminals of each α and β chain, that this terminal was also a Bohr group, that there were three additional Bohr groups per monomer: an aspartic group, a valine and a histidine, and that there were about 200 more oxystable H^+ buffering sites on the tetramer. Tables 2 a,b,c are a listing of the Roughton model.

Although DPG is not present, Roughton did include an inorganic phosphate as a buffer, and he put in Na^+, K^+, Ca^{++}

and Mg^{++} for subsequent protocols. In Table 2a, the input reactants, the usual input species are listed plus the names of the oxy- and deoxy-Bohr sites and the names of the oxystable sites. The latter are entered here at zero levels merely as an accounting tactic; the actual reaction species are shown in Table 2b.

INPUT REACTANTS FOR THE ROUGHTON Hb MODEL		
REACTANTS:	**Solution Moles**	**Partial Press.**
Multipliers:	1.0	1.0 E+05
O2	9.843 E-03	3.500 E-02
CO2	1.787 E-02	5.263 E-02
N2	5.402 E-04	8.505 E-01
H2O	3.859 E+01	6.185 E-02
H+	-2.603 E-01	
NA+	9.667 E-03	
K+	1.019 E-01	
CA++	6.608 E-05	
MG++	1.291 E-03	
CL-	5.368 E-02	
HPO4=	1.404 E-02	
Hb	4.901 E-03	
Deoxy sites		
REDASP SITES	0.0	
ARVAL SITES	0.0	
BRHIS SITES	0.0	
BRVAL SITES	0.0	
Oxy Sites		
OXYASP SITES	0.0	
AOVAL SITES	0.0	
BOHIS SITES	0.0	
BOVAL SITES	0.0	
Oxystable Sites		
DHIST SITES	0.0	
ASPGLU SITES	0.0	
HMCOOH SITES	0.0	
HISTIDINE SI	0.0	
TYROSINE SIT	0.0	
LYSINE SITES	0.0	
ARGININE SIT	0.0	

Table 2a. List of reactants for Hb model devised by F.J.W. Roughton (1972). It is 4.9 millimolar in tetramer (see text for details).

REACTION EQUATIONS FOR ROUGHTON Hb MODEL					
Reaction Products	**Reaction Constant**	**Reactants**			
Gas Phase					
O2	0.026	1. O2			
CO2	0.440	1. CO2			
N2	0.0146	1. N2			
H2O	2.79569	1. H2O			
Hb Solution					
O2	0.	1. O2			
CO2	0.	1. CO2			
N2	0.	1. N2			
H2O	0.	1. H2O			
H+	0.	1. H+			
OH-	13.0958	1. H2O	-1. H+		
NA+	0.	1. NA+			
K+	0.	1. K+			
CA++	0.	1. CA++			
MG++	0.	1. MG++			
CL-	.	1. CL-			
HCO3-	6.13	1. CO2	1. H2O	-1. H+	
HPO4=	0.0	1. HPO4=			
H2PO4-	7.19	1. HPO4=	1. H+		
Hb	.	1. Hb		-8. HMCOOH	-12. TYROSI
		-12. ARGINI	-54. ASPGLU	-16. HISTID	-40. LYSINE
		-4. REDASP	-2. ARVAL	-2. BRHIS	-2. DHIST
		-2. BRVAL			
HbO2	-4.336	1. Hb	1. O2	-8. HMCOOH	-12. TYROSI
		-12. ARGINI	-54. ASPGLU	-16. HISTID	-40. LYSINE
		-3. REDASP	-1. OXYASP	-1. ARVAL	-1. AOVAL
		-2. BRHIS	-2. DHIST	-2. BRVAL	
$Hb(O2)_2$	-8.665	1. Hb	2. O2	-8. HMCOOH	-12. TYROSI
		-12. ARGINI	-54. ASPGLU	-16. HISTID	-40. LYSINE
		-2. REDASP	-2. OXYASP	-2. AOVAL	-2. BRHIS
		-2. DHIST	-2.0BRVAL		
$Hb(O2)_3$	-9.880	1. Hb	3. O2	-8. HMCOOH	-12. TYROSI
		-12. ARGINI	-54. ASPGLU	-16. HISTID	-40. LYSINE
		-1. REDASP	-3. OXYASP	-2. AOVAL	-1. BRHIS
		-1. BOHIS	-2. DHIST	-1. BRVAL	-1. BOVAL
$Hb(O2)_4$	-17.692	1. Hb	4. O2	-8. HMCOOH	-12. TYROSI
		-12. ARGINI	-54. ASPGLU	-16. HISTID	-40. LYSINE
		-4. OXYASP	-2. AOVAL	-2. BOHIS	-2. DHIST
		-2. BOVAL			

Table 2b (continued)

Deoxy Sites				
REDASP	-4.900	1. REDASP	1. H+	
REDAS-		1. REDASP		
BHIS+		1. BRHIS		
BHIS	8.100	1. BRHIS	-1. H+	
AVAL+	-7.700	1. ARVAL	1. H+	
AVAL		1. ARVAL		
AVCO2	11.9770	1. ARVAL	-1. H+	1. CO2
BVAL+	-7.300	1. BRVAL	1. H+	
BVAL		1. BRVAL		
BVCO2	11.764	1. BRVAL	-1. H+	1. CO2
Oxy Sites				
REDASP	-5.500	1. OXYASP	1. H+	
REDAS-		1. OXYASP		
BHIS+		1. BOHIS		
BHIS	7.200	1. BOHIS	-1. H+	
AVAL+	-7.300	1. AOVAL	1. H+	
AVAL		1. AOVAL		
AVCO2	11.280	1. AOVAL	-1. H+	1. CO2
BVAL+	-7.300	1. BOVAL	1. H+	
BVAL		1. BOVAL		
BVCO2	12.100	1. BOVAL	-1. H+	1. CO2
Oxystable Sites				
HMCOOH	-4.000	1. HMCOOH	1. H+	
HMCOO-		1. HMCOOH		
BCOOH	-4.500	1. ASPGLU	1. H+	
BCOO-		1. ASPGLU		
IMID		1. HISTID		
IMID+	-7.000	1. HISTID	1. H+	
DHIST		1. DHIST		
DHIST+	-7.000	1. DHIST	1. H+	
PHENOL		1. TYROSI		
PHENO-	9.800	1. TYROSI	-1. H+	
EAMIN+		1. LYSINE		
EAMIN	10.350	1. LYSINE	-1. H+	
GUAN+		1. ARGINI		
GUANID	12.000	1. ARGINI	-1. H+	

Table. 2b. Reaction products of the model of Hb function devised by Roughton (1972) having three oxylabile (Bohr) protons, CO_2 binding at the N-terminals of each monomer, and 200 oxystable buffering sites.

In the reaction equations, Table 2b, each Hb species, in addition to reacting with O_2, now gives rise to the many H^+ buffering groups, more than 200 per Hb molecule. These groups are actually ionized in an ancillary calculation listed below under oxy-, deoxy-, and oxystable sites (so that their presence does not disturb the solution's mole fraction ratio). Specifically, in each Hb species, there are explicitly mentioned a total of 200 oxystable sites ('carboxyls', tyrosines, arginines, aspartics, histidines, and lysines) followed by 12 oxylabile sites: 4 aspartic groups, 2 α valines, 2 β-valines, 2 β-histidines, and 2 "other" histidines. The valines also bind CO_2.

The titration and Bohr pKs shown for each reaction equation, (Table 2b, column 2) are those determined by fitting observed oxy and deoxy titration curves. The pks for the Hb reactions with O_2 (the a_j), are also determined by fitting a saturation curve using this chemical model as an arbitrary function.

The Roughton oxygenation parameters, a_j, determined in this way, are the log a_j shown opposite the Hb reactions in Table 2b. These parameters do fall within the expected range of modern a_j values (Table 3) even though they might not for two fundamental reasons: recent values are nearly always arrived at using the simpler Adair equation, and, without DPG explicitly in the model aiding the accurate simulation, the a_j values that best fit the curve must accommodate for that absence.

A typical calculation for all of these species in the Roughton model is listed in Table 2c, where the Hb is 50% saturated.

This model could be used to simulate a laboratory H^+ or CO_2 titration protocol. For example, since the oxylabile Bohr sites have different pKs for oxy or deoxy, the calculated titration curves for the reduced molecule will necessarily be different from the oxygenated; the isoionic points are about pH = 7.22 for oxyHb in solution and 7.42 for reduced, and a similar difference will persist from pH 4 to almost 11. Outside of this range the oxystable sites dominate, and the curves intersect. Figure 2 is a plot of the calculated titration experiment.

CALCULATED OUTPUT FOR ROUGHTON Hb MODEL (in moles)					
Products	**Gas Phase**	**Hb Solution**	**Deoxy Sites**	**Oxy Sites**	**Oxystable**
Total Moles	1.0 E+04	3.871 E+01	2.538 E-02	2.363 E-02	7.058 E-01
pH:		7.321			
O2	3.500 E+02	3.922 E-05			
CO2	5.263 E+02	9.447 E-04			
N2	8.505 E+03	5.390 E-04			
H2O	6.185 E+02	3.849 E+01			
H+		4.186 E-08			
OH-		3.090 E-07			
NA+		9.674 E-03			
K+		1.019 E-01			
CA++		6.620 E-05			
MG++		1.292 E-03			
CL-		5.358 E-02			
HCO3-		1.303 E-02			
HPO4=		7.677 E-03			
H2PO4-		6.363 E-03			
Hb		1.589 E-03			
HbO2		8.500 E-04			
$Hb(O2)_2$		4.470 E-04			
$Hb(O2)_3$		3.352 E-07			
$Hb(O2)_4$		2.014 E-03			
REDASP			4.169 E-05	1.638 E-04	
REDAS-			9.762 E-03	9.640 E-03	
BHIS+			5.030 E-03	1.853 E-03	
BHIS			7.432 E-04	2.175 E-03	
AVAL+			2.188 E-03	2.671 E-03	
AVAL			8.120 E-04	2.490 E-03	
AVCO2			1.029 E-03	6.128 E-04	
BVAL+			2.151 E-03	1.768 E-03	
BVAL			2.005 E-03	1.648 E-03	
BVCO2			1.616 E-03	6.128 E-04	
HMCOOH					2.115 E-05
HMCOO-					3.919 E-02
BCOOH					4.509 E-04
BCOO-					2.642 E-01
IMID+					2.751 E-02
IMID					5.092 E-02
DHIST					6.365 E-03
DHIST+					3.438 E-03
PHENOL					5.865 E-02
PHENO-					1.724 E-04
EAMIN+					1.959 E-01
EAMIN					1.623 E-04
GUAN+					5.882 E-02
GUANID					1.091 E-06

Table. 2c. Typical calculated equilibrium distribution of species for the Roughton Hb model at 50% saturation.

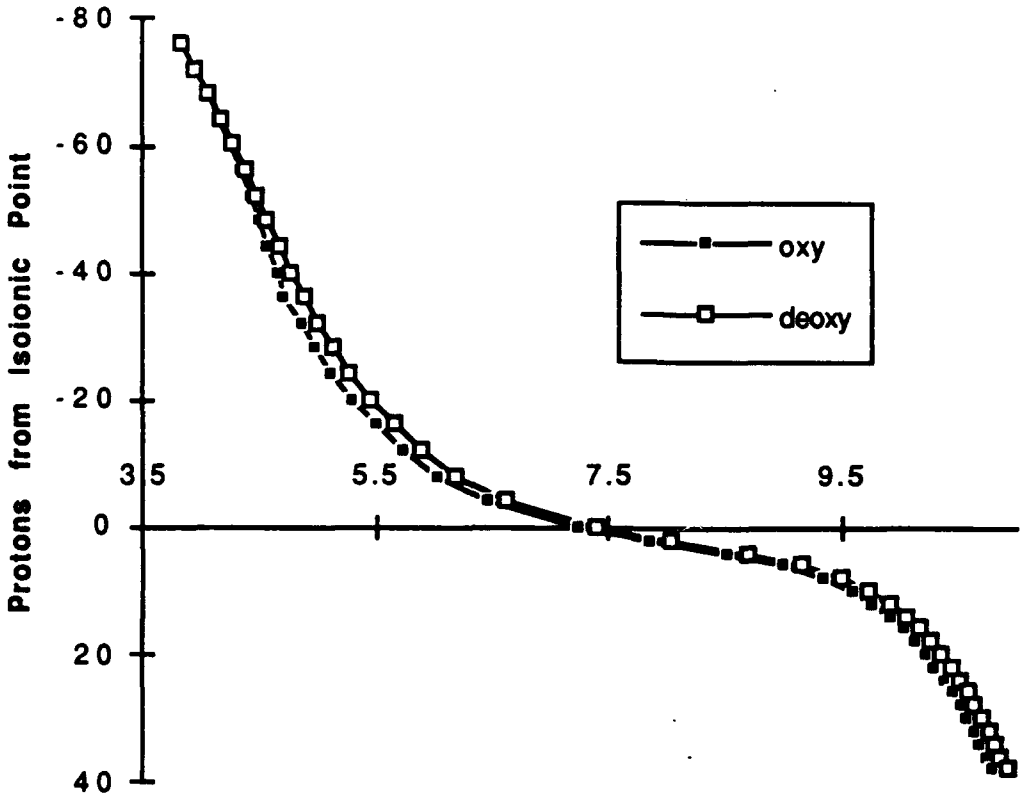

Fig. 2. Titration of Roughton (1972) model; isoionic pH: oxy = 7.25, deoxy = 7.41.

7. The Intrinsic Constants Revisited

Because in this Roughton model the Bohr and carbamino reactions are linked to the oxygenation reaction and it includes the oxystable H^+ buffering, this model is more general than the Adair in the sense that changes in pH and PCO_2 will automatically be accounted for in the model as experimental conditions change. Supposing there were no other affectors, then the model's oxygenation parameters would not need to be changed to simulate changing conditions in the laboratory[38]. This is not yet true since there are other affectors, such as DPG and Cl^-, so that this simple model would fit the data of a laboratory experiment involving DPG only by changing the a_j in a certain way. This is similar to the experience of Winslow, et. al. (Winslow 1983)

fitting the even simpler Adair model to variable sets of laboratory conditions, where they found that the changing a_j were correlated with the log of the affector concentration.

However, supposing for the moment, it were true that the Bohr and Haldane effects were the only affectors and the Roughton hypotheses were correct, then the a_j in the Roughton model would be the intrinsic oxygenation parameters[39]. By "intrinsic", here, we merely mean the binding parameters in the isoionic deoxy Hba0 tetramer in aqueous solution (37° C., PCO2 = 0), not the isolated monomers. These intrinsic parameters are modified to the effective or observed a_j values by competitive interactions with H^+ and CO_2 during oxygenation, just as in the protein.

In fact, this concept can be made mathematically explicit and the degree of modification calculated. The Roughton model can illustrate the process. We wish to take account of the fact that the Bohr and Haldane affectors treat each individual monomer in the tetramer differently depending upon whether they are α or β and whether they are oxy or deoxy.

We first symbolically represent the list of possible product species in solution (from Table 2b). Consider the equations relating only to the first stage of oxygenation:

$$Hb + O_2 \Leftrightarrow HbO_2 , \quad k_1$$

$$Rasp^- + H^+ \Leftrightarrow Rasp , \quad k_{ra}$$

$$\beta Rhis^- + H^+ \Leftrightarrow \beta Rhis , \quad k_{\beta rh}$$

$$\alpha Rval + H^+ \Leftrightarrow \alpha Rval^+ , \quad k_{\alpha rv}$$

$$\alpha Rval - H^+ + CO_2 + \Leftrightarrow \alpha RvCO_2 , \quad k_{\alpha rc}$$

$$\beta Rval + H^+ \Leftrightarrow \beta Rval^+ , \quad k_{\beta rv}$$

$$\beta Rval - H^+ + CO_2 + \Leftrightarrow \beta RvCO_2 , \quad k_{\beta rc}$$

$$Oasp^- + H^+ \Leftrightarrow Oasp , \quad k_{oa}$$

$$\beta Ohis^- + H^+ \Leftrightarrow \beta Ohis , \quad k_{\beta oh}$$

$$\alpha Oval + H^+ \Leftrightarrow \alpha Oval^+ , \quad k_{\alpha ov}$$

$$\alpha Oval - H^+ + CO_2 + \Leftrightarrow \alpha OvCO_2 , \quad k_{\alpha oc}$$

$$\beta Oval + H^+ \Leftrightarrow \beta Oval^+ , \quad k_{\beta ov}$$

$$\beta Oval - H^+ + CO_2 + \Leftrightarrow \beta OvCO_2 , \quad k_{\beta oc}$$

where the chains are α and β, and the O and R stand for oxy and deoxy. From this, we can write the concentration ratio of total oxy sites to deoxy sites (as we did for the Adair equation) and this will allow derivation of the relationship between the intrinsic and observed parameters

$$\frac{HbO_2}{Hb} = k_1' p = \frac{HbO_2 + Oasp + \alpha Ohis + \beta aOval^+ + \beta Oval^+ + \alpha OvCO_2 + \beta OvCO_2}{Hb + Rasp + \alpha Rhis + \beta Rval^+ + \beta Rval^+ + \alpha RvCO_2 + \beta RvCO_2}$$

$$= \frac{k_1 p + k_1 p \frac{H^+}{k_{oa}} + k_1 p \frac{H^+}{k_{\beta oh}} + k_1 p \frac{H^+}{k_{\alpha ov}} + k_1 p \frac{H^+}{k_{\alpha oc}} + k_1 p \frac{H^+}{k_{\beta ov}} + k_1 p \frac{H^+}{k_{\beta oc}}}{1 + \frac{H^+}{k_{ra}} + \frac{H^+}{k_{\beta rh}} + \frac{H^+}{k_{\alpha rv}} + \frac{H^+}{k_{\alpha rc}} + \frac{H^+}{k_{\beta rv}} + \frac{H^+}{k_{\beta rc}}}$$

where we have divided numerator and denominator by Hb and multiplied by 760. Then;

$$k_1' p = k_1 p \frac{1 + \frac{H^+}{k_{oa}} + \frac{H^+}{k_{\beta oh}} + \frac{H^+}{k_{\alpha ov}} + \frac{H^+}{k_{\alpha oc}} + \frac{H^+}{k_{\beta ov}} + \frac{H^+}{k_{\beta oc}}}{1 + \frac{H^+}{k_{ra}} + \frac{H^+}{k_{\beta rh}} + \frac{H^+}{k_{\alpha rv}} + \frac{H^+}{k_{\alpha rc}} + \frac{H^+}{k_{\beta rv}} + \frac{H^+}{k_{\beta rc}}}$$

Thus k_1', the observed constant, is derived from the intrinsic k_1 by the linkage equations. These links are, of course, explicit in the model, and, in the calculation, the ratio of oxy to deoxy sites will be given by k_1'. Similar equations can be derived for subsequent stages of oxygenation but for the jth stage, the right hand side will be raised to the jth power:

$$k_j' p = k_j p \left\{ \frac{1 + \frac{H^+}{k_{oa}} + \frac{H^+}{k_{\beta oh}} + \frac{H^+}{k_{\alpha ov}} + \frac{H^+}{k_{\alpha oc}} + \frac{H^+}{k_{\beta ov}} + \frac{H^+}{k_{\beta oc}}}{1 + \frac{H^+}{k_{ra}} + \frac{H^+}{k_{\beta rh}} + \frac{H^+}{k_{\alpha rv}} + \frac{H^+}{k_{\alpha rc}} + \frac{H^+}{k_{\beta rv}} + \frac{H^+}{k_{\beta rc}}} \right\}^j \qquad (10$$

In a calculation of, say, the saturation curve, these k_j's of Eqn. 10 would replace the implicit k_js in the Adair equation. Following Eqn. 4, we write, using k_j':

$$Hb + jO_2 \Leftrightarrow Hb(O_2)_j \ , k_j', j=1,\ldots,4$$

and since

$$a_i' = \Pi_1^i k_j' \ , i=1,\ldots,4$$

we simply use the a_j' in the Adair equation:

$$y = \frac{a_1'p + 2a_2'p^2 + 3a_2'p^3 + 4a_2'p^4}{4(1 + a_2'p + a_2'p^2 + a_2'p^3 + a_2'p^4)} \qquad (11$$

Now the Adair equation is clearly a function of pH and PCO_2.

Eqn, 11 is the model we have been seeking: the modified Adair with variable a_j. However, while conceptually correct, it is an ungainly procedure to calculate because the H^+ ion concentration is only known in the context of the buffering system and must be calculated separately. It is easier simply to use the chemical model for simulation, where the pH is automatically calculated and introduced.

In a more complicated hypothetical tetramer model, this algebraic relationship is more complicated, but conceptually similar in that each hypothetical configuration of the molecule is represented in the algebra[40]. In this model, the intrinsic parameter is modified primarily by competitive configurations, but the concepts of stereochemical "blocking" or variable local potential fields, allostery, and probability configurations can be included. A possible new approach is to represent the molecular hypotheses in a three dimensional field with chemical reactions and calculate (at least macroscopic) bond energy distributions.

8. Cooperativity

This proposed structure is not yet cooperative. Since Roughton regarded the oxylabile Bohr and Haldane sites to be effective in the vicinity of a single heme no specific linkage was hypothesized among hemes. Roughton was, of course, aware of the changes in tertiary geometry ascribed to the breaking of salt bridges and intramolecular bonds. He

proposed a regulatory mechanism, but lacked the data on Cl^- and DPG. In a more complete model, a mechanisms of linkage must still be established through Cl^-, DPG, and the stereochemistry[41].

This model may nevertheless appear to be cooperative, because the sequential a_j change dramatically, even though Roughton's individual ks are still independent. However, there is some confusion about this point in the literature where, commonly, a_4 is regarded as a measure of the energy of binding of the fourth oxygen ligand (see, for example, Dickerson and Greis[42]), which is, of course, k_4 not a_4. Whereas it may appear that the fourth oxygen is tightly bound because a_4 may be 10^4 times a_1, in fact, the k_j do not exhibit this property so dramatically, if at all. In Hba0, cross-linked ααHb, or blood, the individual k_j are not widely separated; k_2 may be larger or smaller than k_1, and k_4 is generally less than 5 time k_1 and may even be less than k_1 in some circumstances. However, k_3 is generally undetermined by the fitting algorithms usually employed[43] so that k_4 is also frequently inderterminate.

But in analysis of cooperativity and linkage leading to regulation and control of oxygen binding, it is the individual binding parameters k_j that are being operated upon. Generally, the k_j in the tetramer are much smaller than the binding constant for the isolated monomers (1.9 $mmHg^{-1}$ for the α-chain and 3.4 $mmHg^{-1}$ for the βchain[44]). Table 3, (calculated from the data in Winslow 1983 and Vandegriff 1989), shows typical sets of the parameters k_j for pH = 7.4 and various other laboratory conditions.

	DPG	PCO_2	k1	k2	k3	k4
Hba0	0	0	0.326	0.095	k3*k4 = 0.176*	
Hba0	0.4/Hb	0	0.098	0.24	k3*k4 = 0.0158*	
ααHb	0	0	0.124	0.0157	0.154	0.187
ααHb	0.4/Hb	0	0.103	0.0034	0.08	0.392
Blood	1.0/Hb	40	0.022	0.057	0.069	0.023
Blood	0.4/Hb	40	0.012	0.158	0.023	0.050

* a_3 is not available; we use the product of k_3*k_4.

Table 3. Typical literature values for k_j (calculated from a_j values in Winslow 1983 and Vandegriff 1989).

Under the wide conditions of Table 3, the four binding parameters k_j do not differ by more than a factor of about 10 or 20, and considering that the binding parameters for the isolated α and β chains differ by more than a factor of 2, the fraction of the cooperativity to be accounted for in the protein is not great, no more than a factor of 10 in some circumstances. This is the order of magnitude found in simple competitive interaction models, such as the Roughton model.

To illustrate this point, in the literature considerable attention has been devoted to documenting the binding sites and effects of Cl^- in Hb (discussed in Section 10). But in the Marini model (Section 10) Cl^- is merely bound to the terminal amines in competition with CO_2 (which has not been ruled out in the literature). Increasing the Cl- concentration moves the P_{50} for Hba0 in accordance with observed data[45].

In passing, also, it seems clear from these data that DPG, instead of increasing the effective k_4, merely delays oxygenation by reducing k_1. The cross-linking of $\alpha\alpha$Hb, on the other hand, does both, lowering the affinity for oxygen at k_1 and increasing the affinity at k_4. The fact that k_3 is indeterminate is also interesting because of the implication that the third stage of oxygenation may be rare or occur with low probability. Since k_3 is sometimes determinate, as in $\alpha\alpha$HB, the laboratory conditions that alter this probability will shed light on Hb function, e.g., can DPG prevent binding of the third stage until, catastrophically, DPG leaves its pocket and the third and fourth oxygen bind simultaneously?

9. The Adair Derivatives

The fact that the Adair equation can be differentiated has been useful because it yields a guide to the value of k_1. But an interesting innovation is that it also yields a guide to the value of k_2 for Adair-type mathematical models. The first derivative (with the aj constant) is:

```
dy/dp = (a1 + 4*a2*p + 9*a3*p^2 + 16*a4*p^3)/
        (4*(1 + a1*p + a2*p^2 + a3*p^3 + a4*p^4)) -
        (a1 + 2*a2*p + 3*a3*p^2 + 4*a4*p^3)*
        (a1*p + 2*a2*p^2 + 3*a3*p^3 + 4*a4*p^4))/
        (4*(1 + a1*p + a2*p^2 + a3*p^3 + a4*p^4)^2)
```

and the limit of this expression as p -> 0 is:

$$\lim_{p \to 0} (dy/dp) = a_1/4$$

so that near the origin, a_1 , that is k_1, can be calculated from the slope of the saturation curve. As we have argued, this is the observed parameter and it need not be constant over the rest of the curve. But, this may be the intrinsic constant for the first monomer to bind in the tetramer at very small PO_2 since cooperativity, if any, has not yet had an effect.

Also, the function y/(1-y) from the Hill's equation:

```
y/(1-y) = (a1*p + 2*a2*p^2 + 3*a3*p^3 + 4*a4*p^4)/
          (4a0 + 3*a1*p + 2*a2*p^2 + *a3*p^3)
```

yields the same result, as does the derivative of the Roughton chemical model.

The second derivative of the Adair:

```
d2y/dp2  = -((a1 + 2*a2*p + 3*a3*p^2 + 4*a4*p^3)*
           (a1 + 4*a2*p + 9*a3*p^2 + 16*a4*p^3))/
           (2*(1 + a1*p + a2*p^2 + a3*p^3 + a4*p^4)^2) +
           (4*a2 + 18*a3*p + 48*a4*p^2)/
           ((a1 + 2*a2*p + 3*a3*p^2 + 4*a4*p^3)^2*
           (a1*p + 2*a2*p^2 + 3*a3*p^3 + 4*a4*p^4))/
           (2*(1 + a1*p + a2*p^2 + a3*p^3 + a4*p^4)^3) -
           ((2*a2 + 6*a3*p + 12*a4*p^2)
           (a1*p + 2*a2*p^2 + 3*a3*p^3 + 4*a4*p^4))/
           (4*(1 + a1*p + a2*p^2 + a3*p^3 + a4*p^4)^2)
```

which is a measure of the rate of curvature of the saturation curve, also gives an interesting limit near the origin:

$$\lim_{p \to 0} \left(d^2y/dp^2\right) = -a_1^2/2 + a_2 .$$

This curvature near the origin should be positive, that is, the curve turns upward, and therefore the expression is greater than zero, or:

$$a_2 > a_1^2/2 .$$

Near the origin, then, $2a_2$ must be greater than a_1^2. This is a condition not always met by published sets of the a_j. For example, it is not true for all the sets of Table 3. There doesn't appear to be any escape from this condition; the curve does not turn down at the origin. It is obviously

mathematically possible for the first increments of the calculated curve to have negative curvature, but it is not chemically sensible and published detail of the curve near the origin appear to have positive curvature. This illustrates again the fundamental difference between the mathematical Adair equation and the real chemical system, Eqns. 3 ansd 5. Detail of the function of the protein near the origin would be useful.

The third derivative of the Adair does not materially aid this problem. This derivative is the rate of change of he curvature of the saturation curve. Near the origin its value would be non-zero only if the curvature of the sat curve is changing. From detail of the curve, the curvature does appear to increase until about 10 mmHg, and so the limit of the third derivative should be positive. The third derivative is:

d^3y/dp^3 =
```
(3*(a1 + 2*a2*p + 3*a3*p^2 + 4*a4*p^3)^2*
(a1 + 4*a2*p + 9*a3*p^2 + 16*a4*p^3))/
(2*(1 + a1*p + a2*p^2 + a3*p^3 + a4*p^4)^3) -
(3*(4*a2 + 18*a3*p + 48*a4*p^2)*
(a1 + 2*a2*p + 3*a3*p^2 + 4*a4*p^3))/
(4*(1 + a1*p + a2*p^2 + a3*p^3 + a4*p^4)^2) -
(3*(2*a2 + 6*a3*p + 12*a4*p^2)*
(a1 + 4*a2*p + 9*a3*p^2 + 16*a4*p^3))/
(4*(1 + a1*p + a2*p^2 + a3*p^3 + a4*p^4)^2) +
(18*a3 + 96*a4*p)/
(4*(1 + a1*p + a2*p^2 + a3*p^3 + a4*p^4)) -
(3*(a1 + 2*a2*p + 3*a3*p^2 + 4*a4*p^3)^3*
(a1*p + 2*a2*p^2 + 3*a3*p^3 + 4*a4*p^4))/
(2*(1 + a1*p + a2*p^2 + a3*p^3 + a4*p^4)^4) +
(3*(2*a2 + 6*a3*p + 12*a4*p^2)*
(a1 + 2*a2*p + 3*a3*p^2 + 4*a4*p^3)*
(a1*p + 2*a2*p^2 + 3*a3*p^3 + 4*a4*p^4))/
(2*(1 + a1*p + a2*p^2 + a3*p^3 + a4*p^4)^3) -
((6*a3 + 24*a4*p)*(a1*p + 2*a2*p^2 +
   3*a3*p^3+ 4*a4*p^4))/
(4*(1 + a1*p + a2*p^2 + a3*p^3 + a4*p^4)^2)
```

and the limit is:

$$\lim_{p \to 0} (d^3y/dp^3) = (3*a_1^3)/2 - (9*a_1*a_2)/2 + (9*a_3)/2$$

or

$$3*a_1^3 + 9*a_3 > 9*a_1*a_2.$$

Now, while this is a valid result that probably should be satisfied, the standard error of the a_3 determination is larger than the value of either side of the equation.

Finally, it is a curious fact that the isomorphic models we have been discussing also have analytic derivatives, and so a similar analysis of parameters can be made in these more complicated models. The procedure, as with Adair, is to write down a concentration ratio of saturated to unsaturated sites and differentiate this ratio. It has been done only for the simpler models.

10. The Marini-91, Hba0, and X-Linked Models

An isomorphic model can be designed to accommodate a variety of molecular function hypotheses. Another model that has been exceptionally useful is the Marini-91 Model. It differs from the Roughton model primarily in having modern sets of Bohr and Carbamino reactions and pks. In particular, while Roughton confined the Bohr affectors to a few particular groups, Marini-91 proposes that almost all of the H^+ binding sites may be Bohr sites and provides binding parameters for the oxy and deoxy conditions[46]. CO_2 is still bound at the terminal valines, and Cl^- is bound either to the same or another valine; DPG is not represented in these isoionic models.

Since the input reactants for this Hb model change from the Roughton only by dropping Na^+, Ca^{++} and Mg^{++} (see Table 2a), Table 4 is a listing of just the output species for the Marini-91 model. It shows the CO2 and Cl- binding as well as the detail of the Bohr sites with their pKs. The only oxystable sites are 22 Histidine and 8 Tyrosine groups; about 150 groups are oxylabile. This model does not distinguish between the α and β chains.

The Marini-91 model has been used to simulate the functions of Hba0 and cross-linked Hb (XHb). This work is in progress and will not be reported in detail in this note, but some characteristics of the model can be discussed. The buffering power of Hb in this model is very similar to that of the Roughton model since the pKs far from neutrality are inactive in the physiologic range. Although more Bohr sites are buffering in the Marini-91, the relative gaps between oxy and deoxy groups is smaller than in the Roughton model and the differences among the titration curves is, generally, within the precision of the laboratory data. The P_{50} is set at 1 mmHg by fitting the a_j to such a curve. The pH at P_{50} is 7.32. The saturation curve matches the middle data of published curves; detailed data for the ends of the Hba0 curve were not available at this writing.

REACTION EQUATIONS FOR MARINI MODEL

Reaction Products	Reaction Constant	Reactants			
Gas Phase					
O2	0.026	1. O2			
CO2	0.440	1. CO2			
N2	0.0146	1. N2			
H2O	2.7957	1. H2O			
HbaO Solution					
O2	0.0	1. O2			
CO2	0.0	1. CO2			
N2	0.0	1. N2			
H2O	0.0	1. H2O			
H+	0.0	1. H+			
OH-	13.0958	1. H2O	-1. H+		
K+	0.0	1. K+			
CL-	0.0	1. CL-			
HCO3-	6.13	1. CO2	1. H2O	-1. H+	
HPO4=	0.0	1. HPO4=			
H2PO4-	7.19	1. HPO4=	1. H+		
Hb	0.0	1. Hb			
	0.0	-4. C-TERM	-62. ASGLPR	-16. HISTID	-4. CYSTNE
	0.0	-4. N-TERM	-4. TYROSN	-44. LYSINE	-12. ARGINE
	0.0	-22. HISTF	-8. TYRSF		
HbO2	-2.7127	1. Hb	1. O2		
	0.0	-3. C-TERM	-46. ASGLPR	-12. HISTID	-3. CYSTNE
	0.0	-3. N-TERM	-3. TYROSN	-33. LYSINE	-9. ARGINE
	0.0	-1. C-TERO	-15. ASGLPO	-4. HISTIO	-1. CYSTNO
	0.0	-1. N-TERO	-1. TYROSO	-11. LYSINO	-3. ARGINO
	0.0	-22. HISTF	-8. TYRSF		
$Hb(O2)_2$	-5.4086	1. Hb	2. O2		
	0.0	-2. C-TERM	-31. ASGLPR	-8. HISTID	-2. CYSTNE
	0.0	-2. N-TERM	-2. TYROSN	-22. LYSINE	-6. ARGINE
	0.0	-2. C-TERO	-31. ASGLPO	-8. HISTIO	-2. CYSTNO
	0.0	-2. N-TERO	-2. TYROSO	-22. LYSINO	-6. ARGINO
	0.0	-22. HISTF	-8. TYRSF		-3. ARGINE
$Hb(O2)_3$	-8.2291	1. Hb	3. O2		
	0.0	-1. C-TERM	-15. ASGLPR	-4. HISTID	-1. CYSTNE
	0.0	-1. N-TERM	-1. TYROSN	-11. LYSINE	-3. ARGINE
	0.0	-3. C-TERO	-46. 5ASGLPO	-12. HISTIO	-3.0CYSTNO
	0.0	-3. N-TERO	-3. TYROSO	-33. LYSINO	-9.0ARGINO
	0.0	-22. HISTF	-8. TYRSF		
$Hb(O2)_4$	-10.946	1 Hb	4. O2		
	0.0	-4. C-TERO	-62. ASGLPO	-16. HISTIO	-4.0CYSTNO
	0.0	-4. N-TERO	-4. TYROSO	-44. LYSINO	-12.0ARGINO
	0.0	-22. HISTF	-8. TYRSF		

Table 4 (continued)

De-oxy Groups				
CTERM	0.0	1. C-TERM		
CTERC-	2.67	1. C-TERM	-1. H+	
ASGLPR	0.0	1. ASGLPR		
ASGLP-	4.57	1. ASGLPR	-1. H+	
HIST	0.0	1. HISTID		
HIST+	-5.88	1. HISTID	1. H+	
NTRCO2	4.90	1. N-TERM	-1. H+	1. CO2
NTERM	0.0	1. N-TERM		
NTERM+	-7.37	1. N-TERM	1. H+	
NTERCL	-10.97	1. N-TERM	1. H+	1. CL-
CYSTNE	0.0	1. CYSTNE		
CYSTN-	8.92	1. CYSTNE	-1. H+	
TYROSN	0.0	1. TYROSN		
TYROS-	10.27	1. TYROSN	-1. H+	
LYSINE	0.0	1. LYSINE		
LYSIN+	-10.97	1. LYSINE	1. H+	
ARGINE	0.0	1. ARGINE		
ARGIN+	-12.17	1. ARGINE	1. H+	
Hb_Oxy Groups				
CTERM	0.0	1. C-TERO		
CTERC-	2.20	1. C-TERO	-1. H+	
ASGLPR	0.0	1. ASGLPO		
ASGLP-	4.40	1. ASGLPO	-1. H+	
HIST	0.0	1. HISTIO		
HIST+	-5.75	1. HISTIO	1. H+	
NTRCO2	5.01	1. N-TERO	-1. H+	1. CO2
NTERM	0.0	1. N-TERO		
NTERM+	7.16	1. N-TERO	1. H+	
NTERCL	-10.80	1. N-TERO	1. H+	1. CL-
CYSTNE	0.0	1. CYSTNO		
CYSTN-	8.75	1. CYSTNO	-1. H+	
TYROSN	0.0	1. TYROSO		
TYROS-	10.10	1. TYROSO	-1. H+	
LYSINE	0.0	1. LYSINO		
LYSIN+	-10.80	1. LYSINO	1. H+	
ARGINE	0.0	1. ARGINO		
ARGIN+	-12.00	1. ARGINO	1. H+	
Oxystable Groups				
HISTF	0.0	1. HISTF		
HISTF+	-12.070	1. HISTF	+1. H+	
TYRSF	0.0	1. TYRSF		
TYRSF-	29.3395	1. TYRSF	-1. H+	

Table 4. Chemical reactions for the Marini-91 model showing detail of the Bohr, Haldane, and Chloride reactions.

As an example of the use of this model, Cl^- binding was simulated using the suggestions in Chanconne[47] and Haire[48] to locate the binding at the N-terminal valine, also used to bind the CO2. They mention only the αterminal valines (as well as other possible binding sites), but in this first model we merely bind to all four terminal valines. This experiment contained 1.0 mM Hba0 in enough water to make 1 liter. With the Cl^- at 10^{-7} mM, the aj were adjusted to give a P_{50} of 1 mmHg. When the Cl^- is increased to 10 mM, the saturation drops to 40% (at the same pH = 7.32, 37° C.). If the pH is not held constant, the Cl^- binding inhibits the normal H^+ ionization at the site and the resulting increase in pH actually increases saturation. About 2.6 mM of Cl^- was bound to the 1 mM tetramer using the published binding constants. But for these constants the binding reactions are ambiguous, and we believe that this binding and hence the effect on saturation is at least 2 times too strong.

The algebraic relationship between the intrinsic and observed oxygen binding parameters has been derived, but it is similar to that of the Roughton model with additional terms for the additional Bohr sites and the Chloride sites. We are devising a computer program to derive this algebraic relationship and also to calculate its value. That is, we will compute the numerical change in the aj as a consequence of the linkage to the affectors.

11. Conclusion

The Hba0 laboratory experiments run at constant pH, PCO_2, and Cl^-, with zero DPG, and without phosphate buffering can theoretically be approximated reasonably well with the Adair equation. Even blood samples or cross-linked Hb with those constant affectors can be approximated by the Adair. But the constant set of aj found for one species will not work for another, and, within a species, if one of the variables is changed, or pH drifts. a new set of aj will be required. We propose a mathematical model more complicated than the Adair that links the effects of these affectors to saturation in such a way that only one set of aj (the intrinsic aj) are required for general laboratory conditions.

At the same time, such a model is isomorphic in the sense that the detailed linkages are explicitly displayed for analysis and the model functions like, simulates, the protein. The basic concept is that various hypothetical protein structures can be proposed and tested by incorporating their tenets into the mathematical chemistry of a model. The procedures for modeling are not new, they are basic biochemistry, but a novel aspect is that a computer-based

program will support simultaneous management of systems as complex as the Hb protein.

More complicated models, such as the Hb-DPG, red cell, blood, and cross-linked Hb models in solution and in plasma, have been devised and used for a variety of purposes. In particular, experience with the cross-linked Hb models will be published in the next note of this series. This research was supported by the Letterman Army Institute of Research, U.S. Army Medical Research and Development Command, and a Midterm Report dealt specifically with the cross-linked model in physiological experiments. DPG and blood models have been used extensively in this laboratory and have been published elsewhere, though much of that work will be repeated in subsequent notes taking account of modern data.

REFERENCES

[1]This work was supported by the Blood Research Group, Letterman Army Institute of Research, Presidio of San Francisco, Col. R.M. Winslow, Director.

[2]Bohr, C., Zentr. Physiol., Vol. 17, 1903, p. 682.

[3]Dantzig, G.B., J.C. DeHaven, I. Cooper, S.H. Johnson, E.C. DeLand, H.E. Kanter, and C.F. Sams, *A Mathematical Model of the Human Respiratory External System*, Persp. Biol. Med., Vol. IV, No. 3, pp. 324-376, Spring 1961.

[4]White, W.B., S.M. Johnson, and G.B. Dantzig, *Chemical Equilibrium in Complex Mixtures*, J. Chem. Phys., Vol 28, No. 5, May 1958, pp. 751-755.

[5]Nordstrom, D.K., et al. (19 authors), *A Comparison of Computer Methods for Equilibrium Calculations in Aqueous Systems*, Am. Chem. Soc. Symp. #3, Chemical Modeling in Aqueous Systems, I Jenne (Ed.).

[6]Van Zeggren, F. and S.H. Storey, *The Computation of Chemical Equilibrium*, Cambridge University Press, Cambridge, Mass, 1970.

[7]Zelenick, J.E. and S. Gordon, *Analytical Investigations of Three General Methods of Calculating Chemical Equilibrium Compositions*, NASA Technical Report TN-D-473, Washington D.C., 1960.

[8]DeLand, E.C., *The Classical Structure of Blood Biochemistry*, Proc. Alfred Benzon Foundation Symposium IV, Oxygen Affinity of Hemoglobin and Red Cell Acid-Base Status, Munksgaard, Copenhagen, 1971.

[9]Bradham, G.B. and DeLand, E.C., *Isotope Dilution and Thermodynamics in the Study of Intercompartmental Body Fluid Exchange*, Surgery, Gynecology & Obstetrics 119:1062-1068, November 1964.

[10]Hess, J.R., Bangal, N.R., DeLand, E.C., Wade, C.E., and Winslow, R.M., The *Kinetics of Redistribution of aaHb: Two Estimates of the Endothelial Diffusion Coefficient of a Tetrameric Hemoglobin*, (In Publication).

[11]DeLand, E.C., *Validation of Very Large Simulations of Human Biochemistry*, Proc Summer Computer Simulation Conf, Soc. Comp Simulation, 1975, San Francisco, 16 pp.

[12]Vandegriff, K.D., F. Medina, M.A. Marini, and R.M. Winslow, *Equilibrium Oxygen Binding to Human Hb Cross-linked Between Alpha-chains by bis(3,5-dibromosalicyl) Fumarate*, J. Biol. Chem., Vol. 264, 1989, pp. 17824-17833.

[13]Hill, A.V., *The Possible Effects of the Aggregation of the Molecules of Haemoglobin on its Dissociation Curves*, J. Physiol, Vol. 40, 1910, P. IV.

[14]Adair, G.S., *The Hemoglobin System*, J. Biol. Chem., Vol. 63, 1925, pp. 529-545.

[15]Winslow, R.M., J.M. Morrissey, R.L. Berger, P. D. Smith, and C.C. Gibson, *Variability of Oxygen Affinity of Normal Blood: and Automated Method of Measurement*, J. Appl. Physiol: Respirat. Environ. Exercise Physiol., 45(2): 289-297, 1978.

[16]Winslow, R.M., M. Samaja, N.J. Winslow, L. Rossi-Bernardi, R.I. Schrager, *Simulation of Continuous Blood O_2 Equilibrium Curve over Physiological pH, DPG, and PCO_2 Range*, J. Appl. Physiol: Respirat. Environ. Exercise Physiol., Vol. 54, No. 2, 1983, pp. 524-529.

[17]Kilmartin, J.V., *Interaction of Haemoglobin with Protons, CO_2, and 2,3-Diphosphoglycerate*, Br. Med. Bull., Vol. 32, No. 3, 1976, pp. 209-212.

[18]Perutz, N.F.: Annual Review of Biochemistry, Snell, E.E., P.D. Boyer, A. Meister, and C.C. Richardson (Eds.), Vol. 48, 327-386, Annual Reviews, Inc., Palo Alto, 1979.

[19]Kilmartin, J.V., *Influence of DPG on the Bohr Effect of Human Haemoglobin*, FEBS Letters, North Holland Publishing Co., Amsterdam, Vol. 38, No. 2, 1974, pp. 147-148.

[20]Rossi-Bernardi, L., and F.J.W. Roughton, *The Specific Influence of Carbon Dioxide and Carbamate Compounds on the Buffer Power and Bohr Effects in Human Haemoglobin Solutions*, J. Physiol., Vol 189, 1967, pp. 1-20.

[21]Imaizumi, K., K. Imai, and I. Tyuma, *The Linkage between the Four-Step Binding of Oxygen and the Binding of*

Heterotropic Anionic Ligands in Hemoglobin, J. Biochem. Vol 86, 1979, pp. 1829-1840.

[22]Perutz, M.F., M.G. Rosemann, A.F. Cullis, H. Muirhead, G. Will, A.C.T. North, *Structure of Haemoglobin*, Nature, Vol. 185, No. 4711, Feb. 13, 1960, pp. 416-422.

[23]Baldwin, J.M., Prog. Biophys. Mol. Biol., Vol 29, 1975, pp. 225-320.

[24]Baldwin, J.M. and C. Chothia, J. Mol. Biol., Vol 129, 1979, pp. 175-220.

[25]Nasuda-Kouyama, A., H. Yachibana, and A. Wada, *Preference of Oxygenation Between α and β Subunits of Hemoglobin*, J. Molec. Biol., Vol. 164, 1983, pp. 451-476.

[26]Monod, J., J. Wyman, and J. Changeaux, *On the Nature of Allosteric Transitions: A Plausible Model*, J Biol. Chem., Vol. 12, 1965, pp. 88-118.

[27]Pauling, Linus, *The Oxygen Equilibrium of Hemoglobin and its Structural Interpretation*, Proc. N.A.S., Vol. 21, 1935, pp. 186-191.

[28]DiCera, E., C.H. Robert, S.L. Gill: Biochemistry, 26, 4003-4008, 1987.

[29]Ackers, G.K., *Linked Functions in Allosteric Proteins: An Exact Theory of the Effect of Organic Phosphates on Oxygen Affinity of Hemoglobin*, Biochem Vol 18, No. 5, 1979, 3372-3380.

[30]Smith, F.R. and G.K. Ackers, *Experimental Resolution of Cooperative Free Energies for the Ten Ligation states of Human Hemoglobin*, Proc. Nat. Acad. Sci., Vol. 82, 1985, pp. 5347-5351.

[31]Ackers, G.K. and F.R.Smith, *The Hemoglobin Tetramer: A Three-State Molecular Switch for Control of Ligand Affinity*, Ann. Rev. Biophys. Biophys. Chem. Vol.16, 1987, pp. 583-609.

[32]Roughton, F.J.W., F.R.S., E.C.DeLand, J.C. Kernohan, and J.W. Severinghaus, *Some Recent Studies of the Oxyhaemoglobin Dissociation Curve of Human Blood under Physiologic Conditions and the Fitting of the Adair Equation to the Standard Curve*, In Oxygen Affinity Hemoglobin and Red Cell Acid-Base Status, P. Astrup and M. Rorth (Eds.), Academic Press, New York, 1972, pp. 131-146.

[33]Chanutin, A. and R.R. Churnish, *Effects of Organic and Inorganic Phosphates on the Equilibrium of Human Erythrocytes*, Arch. Biochem. Biophys., Vol. 121, 1967, pp. 96-102.

[34]Benesch, R. and R.E. Benesch, *The Effect of Organic Phosphates from the Human Erythrocyte on the Allosteric*

Properties of Hemoglobin, Biochem. Biophys. Res. Com., Vol 26, 1967, pp. 162-164.

[35]Severinghaus, J.W., *Blood Gas Calculator*, J. Appl. Physiol., Vol. 21, 1966, pp. 1108-1118.

[36]Winslow, R.M., J.M. Morrissey, R.L. Berger, P.D. Smith, and C.C.Gibson, *Variability of Oxygen Affinity o Normal Blood: an Automated Method of Measurement*, J. Appl. Physiol.: Resp. Environ.. Exercise Physiol, Vol 42, No. 2, 1978, pp. 289-297.

[37]Winslow, R.M., M. Samaja, N.J. Winslow, L. Rossi-Bernardi, and R. Shrager, Simulation of Continuous Nlood O_2 Equilibrium Curve over Physiological pH, DPG, and PCO_2 Range, J. Appl. Physiol: Resp. Environ. Exercise Physiol, Vol. 54, No. 2, 1983, pp. 524-529.

[38]Of course, this discussion presumes that the molecular hypotheses upon which the model was constructed were correct. But, the Roughton model was devised years ago, and better data is now available (Roughton's a_j do not agree with modern values) along with more sophisticated conjectures concerning the complex interactions of Hb.

[39]Assuming that the oxygenation k_j for each of the monomers in the tetramer are nearly equal, the intrinsic a_j would be nearly uniform multiples of the first parameter k_1, which they nearly are in the Roughton model (the pKs, that is, the log of the a_j are an additive progression, Table 2b). Sets of parameters in the literature could have this property also if the affectors are held constant as in Winslow (1983) and Vandegriff (1989). The fact that they do not indicates the degree of naivete in the Roughton model.

[40]A fundamental problem exits in this regard: many of the reaction parameters, such as the H^+ ionization pKs, are gathered by observation of the protein function in the laboratory. Hence these parameters are observed or effective parameters, as modified in context, perhaps calculated from the heats of ionization, for example. Whereas, just like the oxygen parameters, all reaction constants in the model should have the intrinsic values which are then modified in context, just as in the protein. Frequently, as in Roughton's case, observed pKs are used simply because nothing else is available.

[41]The set of a_j from the Roughton model

[42]Dickerson, R.E. and I. Geis, *Hemoglobin: Structure, Function, Evolution, and Pathology*, Benjamin Cummings Publishing Co., 1983, (see for example, page 23).

[43]Calculations of the k_4 from the measured a_j are usually conjectural because of an artifact in the process. When

fitting the Adair (A) to the observed Saturation curve (S), the partial derivative of the error, which we may call $\Sigma(A-S)^2$, with respect to a_3 is essentially zero. That is, the process of fitting the curve is insensitive to and hence ambivalent about the value of a_3; a_3 can be set arbitrarily without affecting the goodness of the fit. The fact that a_3 is frequently found to be near zero does not necessarily imply the absence of the third stage of oxygenation from the reaction mixture. Parameter a_3 still plays an important role in the real chemistry, of course, its value just can not be determined in the usual manner. If, in the Adair equation, the a_j were replaced by products of the k_j during the fitting process, the procedure would give satisfactory results for the k_j (including k_3), but calculation of the sensitivity coefficients is considerably more complicated. This result for this problem not obvious from the examination of the partial derivative with respect to a_3, which is:

$$\frac{\partial A}{\partial a_3} = (3*p\text{^}3)/(4*(1 + a1*p + a2*p\text{^}2 + a3*p\text{^}3 + a4*p\text{^}4)) - (p\text{^}3*(a1*p + 2*a2*p\text{^}2 + 3*a3*p\text{^}3 + 4*a4*p\text{^}4))/(4*(1 + a1*p + a2*p\text{^}2 + a3*p\text{^}3 + a4*p\text{^}4)\text{^}2),$$

but during calculation, either the analytic or numerical derivative of the error function is nearly zero with respect to a_3. This problem does not always occur, but it is not known why, in some cases, a3 can be determined. A conjecture is that if a_3 is indeterminate, the third stage of oxygenation is, in fact, improbable in the reaction mixture, but this has not been proved.

[44]Baldwin 1979, ibid.

[45]R. M. Winslow and R. L. Berger, (private communication).

[46]Marini, M., Letterman Army Institute of Research, Presidio, San Francisco, Private communication.

[47]Chanconne, E., J.E. Norne, S. Forsen, J. Bonaventura, M. Brunori, E. Antonini, and J. Wyman, *Identification of Chloride Binding Sites in Hemoglobin by Nuclear-Magnetic-Resonance Quadrupole-Relaxation Studies of Hemoglobin Digests*, Eur. J. Biochem., Vol. 55, 1975, pp. 385-390.

[48]Haire, R.N. and Bo E. Hedlund, Proc. Nat. Acad. Sci., USA, Vol. 74, No. 10. 1977, pp. 4135-4138.